Kallikrein-Related Peptidase 8–Dependent Skin Wound Healing Is Associated with Upregulation of Kallikrein-Related Peptidase 6 and PAR2

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Kallikrein-related peptidase 8 (KLK8) is believed to be involved in the maintenance of skin homeostasis and pathogenesis of inflammatory skin diseases. Although previous studies have shown that KLK8 is expressed around incisional wounds, the exact role of KLK8 in wound healing remains obscure. In the present study, we compared wound healing in wild-type (WT) and *Klk8* gene–disrupted (kallikrein-related peptidase 8 knockout, $Klk8^{-/-}$) mouse skin. Wound healing in $Klk8^{-/-}$ mice was hampered with defective keratinocyte proliferation, differentiation, and migration in the early stages of wound healing. Compared with the prominent induction of Klk6 and protease-activated receptor 2 (PAR2) messenger RNA, and protein in WT mice after wounding, a much lower increase was observed in $Klk8^{-/-}$ skin. After skin wounding in WT mice, increased Klk6 was detected from the upper stratum spinosum to the stratum corneum. Moreover, in WT mice, Klk6 protein was processed. PAR2 was diffusely expressed in the cytoplasm of the stratum spinosum at day 7 post wounding in WT mice. These results suggest that Klk8 is involved in the proliferation and migration of keratinocytes through the upregulation and activation of Klk6 in the early stages of wound healing, and possibly in keratinocyte differentiation associated with the upregulation and activation of PAR2 in the late stages of wound healing.

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INTRODUCTION

Several proteases of the kallikrein family are expressed in the epidermis and are involved in the maintenance of structural integrity and barrier function of the epidermis. Aberrations in human tissue kallikrein (KLK) activity have been detected in the skin diseases characterized by an abnormal epidermal barrier such as atopic dermatitis (Komatsu *et al.*, 2007).

KLKs comprise a subgroup of 15 serine proteases encoded by a tightly clustered multigene family on chromosome 19q13.4 (Evans *et al.*, 1987; Yousef *et al.*, 2000). This region is also synthenic to the locus on mouse chromosome 7 where the mouse kallikrein gene family cluster is localized (Evans *et al.*, 1987; Yoshida *et al.*, 2000), with mouse serine proteases sharing a high degree of sequence and structural similarity with human homologs (Evans *et al.*, 1987; Yousef *et al.*, 2000). According to the current nomenclature, KLK1 is a tissue kallikrein, and the other 14 KLKs are kallikrein-related peptidases. The importance of KLKs in the skin is implicated by the complex mechanisms that regulate KLKs in the epidermis. KLKs, including KLK7 and KLK8, are transported by lamellar granules and secreted into intercellular spaces between the stratum granulosum and stratum corneum (Ishida-Yamamoto *et al.*, 2004), followed by activation via an activation cascade (Eissa and Diamandis, 2008). KLK5 is believed to initiate the cascade reaction by autoactivation and activation of KLK7 and KLK14; the latter then activates KLK5, creating a positive feedback loop (Emami and Diamandis, 2008). KLK5 and KLK7 can proteolyze corneodesmosomal proteins, leading to corneocyte shedding (Caubet *et al.*, 2004). Other KLKs involved in the proteolysis of corneodesmosomes include KLK1, KLK6, and KLK14, which cleave desmoglein 1 (Borgono *et al.*, 2007). Importantly, KLKs can activate protease-activated receptors (PARs; Oikonomopoulou *et al.*, 2006; Stefansson *et al.*, 2008).

Human KLK8 was originally cloned from skin cDNA as the homolog of mouse Klk8/neuropsin (Klk8). Its cDNA and predicted amino-acid sequence have 72% identity to mouse neuropsin, and key amino-acid residues for enzyme activity are conserved between humans and mice (Yoshida *et al.*, 1998). Among the trypsin-like KLKs, KLK8 is the most abundant in the stratum corneum and sweat (Komatsu *et al.*, 2006).

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Abbreviations: AP-2α, activating protein-2α; HGF, hepatocyte growth factor; KLK, kallikrein-related peptidase; Klk, mouse kallikrein–related peptidase; $Klk8^{-/-}$, kallikrein-related peptidase 8 knockout; PAR2, protease-activated receptor 2; WT, wild type

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Previous reports suggest that KLK8 is upregulated in inflammatory skin diseases and wound healing (Kitayoshi *et al.*, 1999; Komatsu *et al.*, 2007). Furthermore, our previous study suggests that Klk8 is also involved in desquamation in 12-O-tetradecanoyl-phorbol acetate–exposed skin through cleavage of desmoglein 1 and corneodesmosin, possibly in cooperation with other Klks (Kishibe *et al.*, 2007). Recently, Eissa *et al.* (2011) reported that KLK8, activated by KLK5, can process pro-KLK1 and pro-KLK11 *in vitro*. The role of KLK8 in the activation cascade and its function *in vivo* remains to be fully elucidated.

PARs are G-protein-coupled, seven transmembrane–spanning membrane receptors present in many cell types including keratinocytes (Dery *et al.*, 1998). PARs are activated by cleavage of the extracellular N-terminal domain of the receptor, releasing a small peptide that activates the receptor as a tethered ligand. The activation of PAR2 by KLKs in keratinocytes has received much attention. PAR2 has been implicated in the regulation of keratinocyte proliferation and differentiation (Derian *et al.*, 1997; Macfarlane *et al.*, 2005), epidermal barrier homeostasis (Hachem *et al.*, 2006), and inflammation (Briot *et al.*, 2010). KLK5, KLK6, and KLK14, but neither KLK7 nor KLK8, activate PAR2 *in vitro* (Oikonomopoulou *et al.*, 2006; Stefansson *et al.*, 2008). Gene mutation of the serine protease inhibitor Kazal-type 5 (SPINK5) causes hyperactivity of KLK5, resulting in excessive desquamation, upregulation, and activation of PAR2, and induction of various inflammatory cytokines (Briot *et al.*, 2009). Therefore, methods that control KLK-induced PAR2 signaling might offer new therapeutic strategies for the skin barrier dysfunction and inflammatory skin diseases.

To elucidate the physiological functions of KLK8 in the skin, we analyzed wound healing in kallikrein-related peptidase 8 knockout ($Klk8^{-/-}$) mice and found that it was significantly delayed and was associated with upregulation of other Klks and marked inhibition of PAR2.

RESULTS

Klk8 mRNA and protein were upregulated after skin wounding

We first investigated Klk8 messenger RNA (mRNA) expression during skin wound healing. Although relatively weak expression of the *Klk8* gene was detected before wounding, it was markedly induced after wounding (Figure 1a). Quantitative reverse transcriptase–PCR (RT-PCR) also revealed that Klk8 mRNA was upregulated from days 1–14 post wounding, peaking at day 7. The maximal level was 11-fold greater than that before wounding (Figure 1b). Klk8 immunoreactivity was weak in the stratum granulosum at day 1 post wounding, but became markedly intense in the region from the lower stratum spinosum to the stratum corneum in the proliferating epidermis until day 7 (Figure 1c-f).

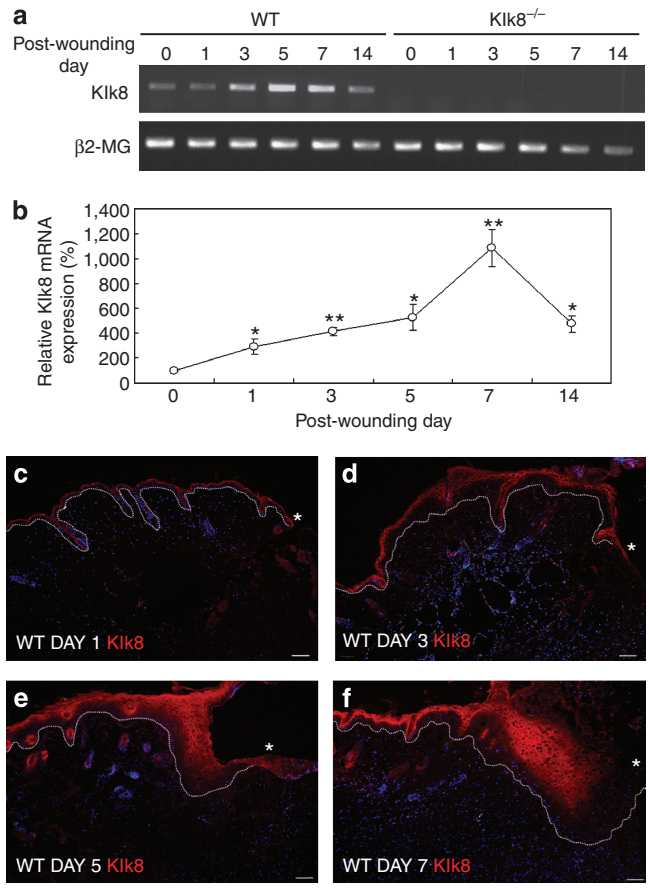

Figure 1. Expression of Klk8 messenger RNA (mRNA) in skin wounds of wild-type (WT) and $Klk8^{-/-}$ mice. (**a**) Klk8 mRNA in the wounded skin of WT and $Klk8^{-/-}$ mice. (**b**) Real-time reverse transcriptase–PCR (RT-PCR) analysis of Klk8 mRNA expression in the wounded skin of WT mice ($n=6$). Data are presented as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ indicate significant difference from day 0. Immunohistochemistry for Klk8 (red) with 4′, 6-diamino-2-phenylindole (DAPI) staining (blue) at days (**c**) 1, (**d**) 3, (**e**) 5, and (**f**) 7 post wounding in the WT mice. The dermoepidermal junction is demarcated by dotted lines. Asterisks demarcate the migrating wound edge. Bars = 50 μm. DAPI, 4′, 6-diamino-2-phenylindole; DAY, post-wounding day; β2-MG, β2-microglobulin.

Wound healing in $Klk8^{-/-}$ mice was significantly delayed compared with WT mice

To assess the role of Klk8 in wound healing *in vivo*, we measured wound sizes daily until complete epithelialization in wild-type (WT) and $Klk8^{-/-}$ mice. Macroscopically, wound sizes were larger in $Klk8^{-/-}$ mice (Figure 2a). Daily measurements revealed a significant difference in wound sizes between the two genotypes from day 3 to day 11 post wounding (Figure 2c). In addition, the wound closure time of $Klk8^{-/-}$ mice was significantly longer than that of WT mice (14.5 ± 1.8 days vs. 13.0 ± 1.2 days, $P<0.05$). Figure 2b shows representative micrographs of wounded skin at day 7 post wounding, showing a larger wound gap in $Klk8^{-/-}$ mice compared with WT mice. These findings suggest that *Klk8* gene deletion delays wound healing.

Impact of Klk8 deletion on wound healing

Wound healing is a complex event involving the coordination of epidermal proliferation, migration, differentiation, and angiogenesis. We compared these changes in WT and $Klk8^{-/-}$ skin. Microscopic observation revealed that the re-epithelialized epidermis was grossly thicker in WT mice than in $Klk8^{-/-}$ mice from days 3 to 7 post wounding (Figure 3a, b,

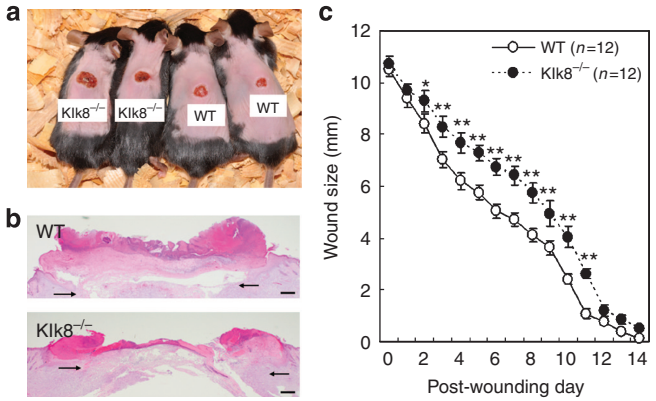

Figure 2. Delay in cutaneous wound healing in Klk8$^{-/-}$ mice.
(**a**) Representative appearance of wounds at day 5. (**b**) Hematoxylin and eosin (H&E) staining of wounded skin at day 7 post wounding. Arrows demarcate the wound margins. Bars = 300 μm. (**c**) Maximum length of the wound in wild-type (WT; open circles) and Klk8$^{-/-}$ mice (filled circles) post wounding ($n = 12$, for each time point and group). Data are presented as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$.

d, and e), and this was confirmed by the measurements taken at days 3 and 5 post wounding (Figure 3g). Closer examination of the stratum granulosum of the re-epithelialized epidermis in Klk8$^{-/-}$ mice at day 7 showed increased cellular volume, with cells containing coarse and abundant keratohyaline granules; this feature was seen only in a single layer of cells in the skin of WT mice (Figure 3 b, c, e, and f, arrows). The stratum granulosum in Klk8$^{-/-}$ mice was significantly thicker than in WT mice (97.0 ± 12.3 μm vs. 37.1 ± 7.7 μm, $n = 5$, $P<0.01$). These findings suggest slower keratinocyte differentiation in Klk8$^{-/-}$ mice. We also measured the distance between the migrating epidermal tip and the original wound edge. The length of the migrating epidermis of WT mice was greater than that of Klk8$^{-/-}$ mice at day 3 post wounding, but no significant difference was observed at day 5 (Figure 3h).

We next compared the number of Ki-67-positive cells after wounding to assess keratinocyte proliferation in WT and Klk8$^{-/-}$ mice (Figure 3i). In WT mice, the number of Ki-67-positive cells was increased, with a peak observed 3 days after wounding. However, the increase in Ki-67-positive cells was delayed in Klk8$^{-/-}$ mice with a peak observed 5 days after wounding. WT skin showed significantly more Ki-67-positive cells at days 1 and 3 post wounding. These results suggest that Klk8 is involved in the proliferation and migration of keratinocytes at the early stages of wound healing. The delayed re-epithelialization in Klk8$^{-/-}$ mice might be a result of decreased keratinocyte proliferation.

The number of CD31-positive vessels, indicative of angiogenesis, was not significantly different between WT and Klk8$^{-/-}$ mice (Figure 3j).

The expression of activating protein-2α (AP-2α) mRNA, which is inhibited by KLK8 (Shingaki *et al.*, 2010), was more suppressed until day 1 post wounding and was more upregulated after day 3 in WT mice, although there was no statistical significance (Supplementary Figure S1 online). In addition, AP-2α protein was more abundant in WT at day 5.

Inflammatory cytokines including tumor necrosis factor-α, IL-1β, IL-6, and hepatocyte growth factor (HGF), and c-Met receptor, which is activated by HGF (Yoshida *et al.*, 2003), were compared between two genotypes (Supplementary Figure S2 online). Tumor necrosis factor-α, IL-1β, and c-Met mRNA in WT were significantly more upregulated than that in Klk8$^{-/-}$ mice at day 1 post wounding. IL-6 mRNA was significantly increased in WT at day 1, although there was no significant difference between genotypes. HGF in WT mice was significantly increased; however, its expression level in WT was lower at day 3 post wounding and higher than that in Klk8$^{-/-}$ at days 5 and 7.

Changes in the expression of Klk6 mRNA and their products in WT and Klk8$^{-/-}$ skin after wounding

As Klks function in a cascade reaction involving multiple members, we investigated the expression of Klk6 and Klk7 in WT and Klk8$^{-/-}$ epidermis after skin wounding. Quantitative RT-PCR revealed that Klk6 mRNA was increased in both genotypes post wounding relative to day 0, with significantly greater upregulation in WT mice (Figure 4a). Klk6 mRNA was increased up to 40-fold in WT mice, with the peak at 3 days after wounding, whereas the skin of Klk8$^{-/-}$ mice showed only a 20-fold increase with the peak at day 1. Klk7 mRNA was upregulated 5-fold at 5 days after skin wounding in WT mice, and increased Klk7 mRNA expression was also observed in Klk8$^{-/-}$ skin, but this was not statistically significant (Supplementary Figure S3 online). These results suggest that Klk8 is involved in the induction of Klk6 (and perhaps Klk7) mRNA after skin wounding.

We next investigated the expression of Klk6 proteins in wounded mice skin. Immunohistochemistry revealed that Klk6 was weak in the epidermis, but was prominent in the hair follicles in both genotypes before wounding (data not shown). After skin wounding, Klk6 increased steadily in the upper stratum spinosum and stratum corneum in WT mice until day 3 post wounding (Figure 4b, d, and h), after which expression subsided but remained detectable at day 7 (Figure 4f and j). Colocalization of Klk8 and Klk6 was observed on the cell surface or in the intercellular spaces of the stratum spinosum and stratum corneum at day 7 post wounding in WT mice (Figure 4f and j). In contrast, Klk6 expression in Klk8$^{-/-}$ mice was confined to the thin layers of the upper epidermis after wounding (Figure 4c, e, g, i, and k).

Western blot analysis detected a greater increase in Klk6 expression in WT mice than in Klk8$^{-/-}$ mice during wound healing (Figure 4l). Molecular sizes of Klk6 in WT appeared smaller than those in Klk8$^{-/-}$ mice, suggesting processing of Klk6 after wounding in WT skin.

Changes in the expression of PAR2 mRNA and its products in WT and Klk8$^{-/-}$ skin after wounding

As Klk6 could directly activate PAR2 (Oikonomopoulou *et al.*, 2006), we investigated the expression of PAR2 in the wounded skin of WT and Klk8$^{-/-}$ mice. Although PAR2

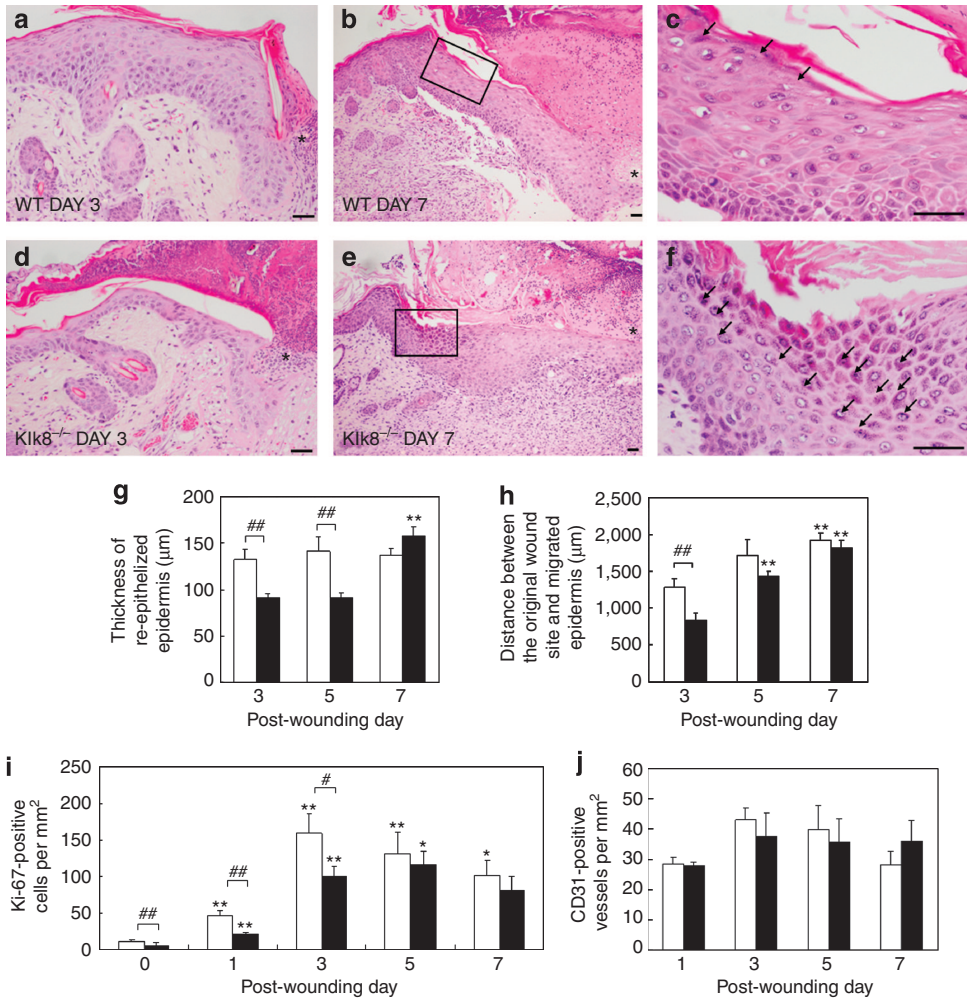

Figure 3. Histopathological examination of skin wounds in (wild-type) WT and Klk8$^{-/-}$ mice. (**a**–**f**) Hematoxylin and eosin (H&E) staining of wounded skin. Panels **c** and **f** are higher magnification of boxed areas in **b** and **e**, respectively. Arrows indicate stratum granulosum keratinocytes containing coarse and abundant keratohyaline granules. Asterisks indicate migrating wound tips. Bar = 50 μm. DAY, post-wounding day. (**g**) Thickness of the re-epithelialized epidermis of WT (white bar; $n = 6$) and Klk8$^{-/-}$ mice (black bar; $n > 5$) after wounding. (**h**) The distance from the original wound site to the migrating wound tip in WT (white bar; $n = 7$) and Klk8$^{-/-}$ mice (black bar; $n = 7$). (**i**) Changes in the number of Ki-67-positive keratinocytes of WT (white bars; $n = 4$) and Klk8$^{-/-}$ mice (black bars; $n = 4$) after wounding. (**j**) The number of CD31-positive vessels within a 1-mm^2 area from the migrating wound tip. Data are presented as mean ± SEM. (**g**, **h**) * And ** indicate a significant difference from day 3 post wounding or (**i**) from the day without wounds ($P < 0.05$ and $P < 0.01$, respectively); $^{\#}$ and $^{\#\#}$ indicate a significant difference between the two genotypes ($P < 0.05$ and $P < 0.01$, respectively).

mRNA was increased after wounding in both genotypes (Figure 5a), the increase of PAR2 mRNA in WT skin was markedly more compared with Klk8$^{-/-}$ skin. Immunohistochemistry showed that PAR2 was weak and confined to the granular layer at day 1 post wounding (Figure 5b and c), but steadily increased in the proliferating epidermis until day 7 in both genotypes (Figure 5d–g). At days 3 and 7 post wounding, membranous PAR2 immunoreactivity was detected in the stratum granulosum and was diffuse in the cytoplasm in the lower stratum spinosum in WT mice (Figure 5h and j). In contrast, in Klk8$^{-/-}$ mice, PAR2 was localized on the cell membrane in the stratum granulosum (Figure 5i, and k). As PAR2 is internalized following its activation by ligand proteases (Ossovskaya and Bunnett, 2004), our results suggest that PAR2 was more induced and activated in WT than in Klk8$^{-/-}$ mice.

DISCUSSION

In this study, skin wound healing was delayed in Klk8$^{-/-}$ mice, suggesting an important role of Klk8 in this process. Previous studies showed that KLK8 was expressed around the incisional wound made in the central nervous system (Tomizawa *et al.*, 1999) and in the human and mouse skin (Kitayoshi *et al.*, 1999; Kuwae *et al.*, 2002). Consistent with these findings, we observed an increase in Klk8 mRNA, as well as an increase in Klk8 protein in the stratum spinosum and corneum of the proliferating epidermis, but not in the migrating tip, after wounding (Figure 1). Kitayoshi *et al.* (1999) also reported that Klk8 was not expressed in the migrating wound tip during re-epithelialization, suggesting that Klk8 might accelerate differentiation rather than affect proliferation or migration of keratinocytes. Keratinocytes in the wounded margins begin to proliferate behind the actively

migrating region during days 1–2 post injury (Singer and Clark, 1999). In the present study, keratinocyte proliferation was significantly increased in WT mice compared with Klk8$^{-/-}$ mice in the relatively early stages of wound healing (1–3 days post wounding), suggesting an involvement of Klk8 in epidermal proliferation. A recent study indicated that KLK8 inhibits expression of the transcription factor, AP-2a (Shingaki *et al.*, 2010), which is involved in terminal differentiation and the loss of AP-2α representing hyperproliferation (Wang *et al.*, 2006). Our findings (Supplementary Figure S1 online), combined with these reports, suggest that Klk8 may accelerate keratinocyte proliferation in the early stages of wound healing, and differentiation in the late stage of wound healing. We also found that upregulation of tumor necrosis factor-α and IL-1β, which is important for normal wound healing (Hubner *et al.*, 1996), was suppressed in Klk8$^{-/-}$ mice (Supplementary Figure S2 online), suggesting that Klk8 promotes normal wound healing associated with inflammatory cytokines.

Hypergranulosis (thickening of the stratum granulosum) was observed in the migrating epidermis in Klk8$^{-/-}$ mice at day 7 post wounding (Figure 3e and f), possibly because of accelerated differentiation of keratinocytes in the stratum spinosum and/or slow transformation of granular keratinocytes into corneocytes in the re-epithelialized epidermis. Previously, we reported that Klk8 is involved in corneocyte shedding through cleavage of desmoglein 1 and corneodesmosin in association with other Klks, including Klk6 (Kishibe *et al.* 2007). These data suggest that Klk8 may have an important role in regulating epidermal differentiation. Interestingly, the level of keratinocyte proliferation and migration in Klk8$^{-/-}$ mice appear to approach that of WT mice after day 5 post-wounding (Figure 3g–i). The reduction in wound size appeared to also follow this trend; wounds in Klk8$^{-/-}$ mice were larger in the early days after wounding, but their size approached that of WT mice after day 7 (Figure 2c). These observations suggest that compensatory mechanisms such as activation of other proteases exist at later stages of wound healing.

As the cascade reaction of KLKs influences the various functions of KLKs, we investigated the relationship between Klk8 and other Klks in wound healing (Caubet *et al.*, 2004; Brattsand *et al.*, 2005). KLK5 is believed to be the initiator of the activation cascade (Caubet *et al.*, 2004; Brattsand *et al.*, 2005); however, we were unable to detect Klk5 in this study, possibly because of its low expression in mouse skin. As was seen in our previous 12-O-tetradecanoyl-phorbol acetate–application study (Kishibe *et al.* 2007), Klk8 mRNA was upregulated in parallel with Klk6 and Klk7 mRNA (Figure 4a

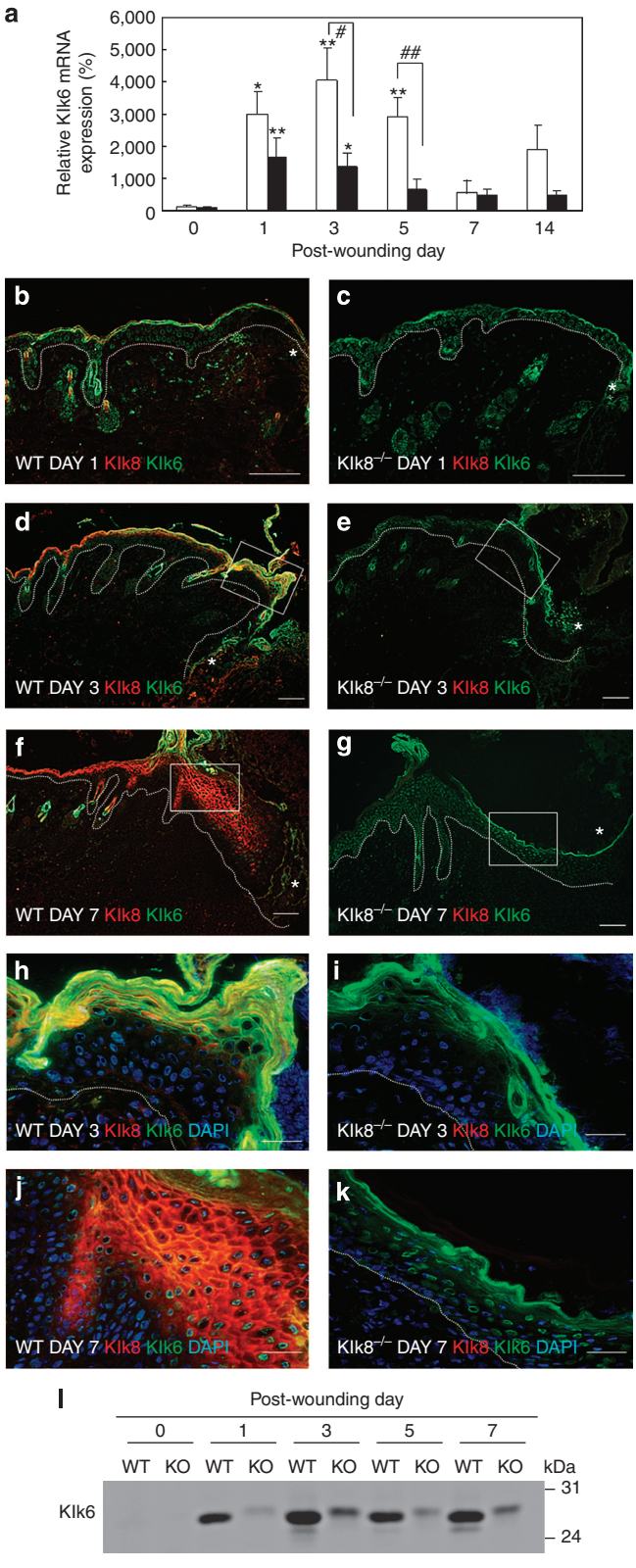

Figure 4. Expression of Klk6 after wounding in wild-type (WT) and Klk8$^{-/-}$ mice. (**a**) Real-time PCR of Klk6 mRNA. White bars, WT mice ($n>5$). Black bars, Klk8$^{-/-}$ mice ($n>5$). Data are presented as mean ± SEM. * And ** indicate a significant difference compared with day 0 ($P<0.05$ and $P<0.01$, respectively); $^{\#}$ and $^{\#\#}$ indicate a significant difference between the two genotypes ($P<0.05$ and $P<0.01$, respectively). (**b–k**) Expression of Klk6 (green) and Klk8 (red) at (**b**, **c**) days 1, (**d**, **e**) 3, and (**f**, **g**) 7 after wounding in WT (left panels) and Klk8$^{-/-}$ mice (right panels). **h**, **i**, **j**, and **k** are magnified images of the boxed insets in **d**, **e**, **f**, and **g**, respectively, with DAPI staining (blue). Asterisks indicate the direction of the migrating wound tip. The dermoepidermal junction is demarcated by the dotted lines. (**b–g**) Bars = 50 μm or (**h–k**) 20 μm. DAY, post-wounding day; DAPI, 4′, 6-diamino-2-phenylindole. (**l**) Western blot analysis of Klk6 proteins in WT and Klk8$^{-/-}$ mice after wounding.

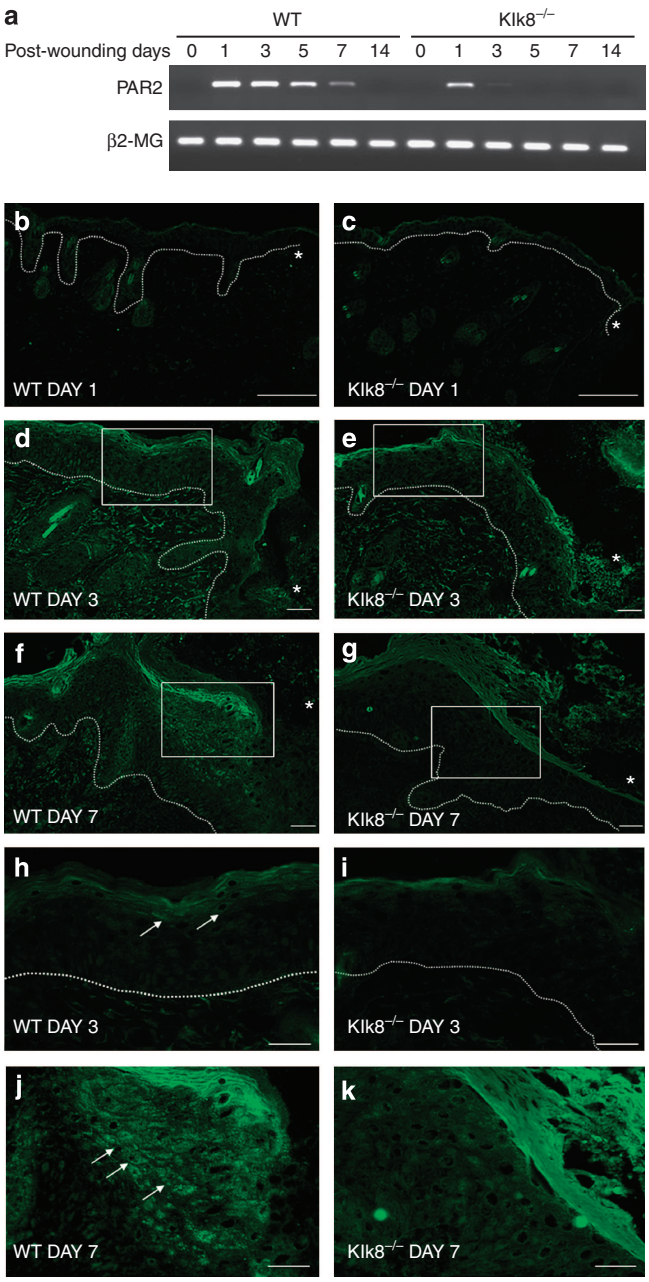

Figure 5. PAR2 expression after wounding in wild-type (WT) and Klk8$^{-/-}$ mice. (**a**) Reverse transcriptase–PCR (RT-PCR) analysis of proteinase-activated receptor 2 (PAR2) mRNA expression in the wounded skin of WT and Klk8$^{-/-}$ mice. (**b–k**) Expression of PAR2 (green) at (**b**, **c**) days 1, (**d**, **e**) 3, and (**f**, **g**) 7 post wounding in WT (left panels) and Klk8$^{-/-}$ mice (right panels). **h**, **i**, **j**, and **k** are magnified images of the boxed insets in **d**, **e**, **f**, and **g**, respectively. The asterisks indicate the direction of the migrating wound tip. Arrows indicate cytoplasmic staining for PAR2. The dermoepidermal junction is demarcated by dotted lines. (**b–g**) Bars = 50 μm or (**h**, **k**) 20 μm. DAY, post-wounding day, β2-MG, β2-microglobulin; PAR2, proteinase-activated receptor 2.

and Supplementary Figure S3 online), suggesting that these enzymes function in the activation cascade. The expression of Klk7 was not significantly different between the two genotypes, whereas Klk6 mRNA was upregulated to a greater degree in WT mice. These findings suggest a close relationship between Klk8 and Klk6, as was observed in the epidermis after 12-O-tetradecanoyl-phorbol acetate treatment, but the mechanism of Klk6 and Klk8 mRNA upregulation remains unknown. As Klk8 transfection of Klk8$^{-/-}$ keratinocytes did not induce Klk6 mRNA expression in our previous study (Kishibe *et al.* 2007), Klk8 itself does not seem to be involved in the transcriptional regulation of Klk6, at least *in vitro*. Western blot analysis revealed that the 27.5-kDa Klk6 was detected in WT mice, whereas the 28.2-kDa Klk6 was detected in Klk8$^{-/-}$ mice. This 0.7 kDa difference between two genotypes is consistent with the size of processed pro-sequence. Therefore, we speculate that Klk8 can activate Klk6 either directly or indirectly. During cutaneous wound healing, the keratinocytes of Klk6 transgenic mice showed increased proliferation and migration associated with reduced E-cadherin protein compared with control littermates (Klucky *et al.*, 2007). Similarly, the differences in keratinocyte migration and proliferation in the early stages of wound healing in this study may be influenced by Klk6.

Evidence of the involvement of PAR2 in wound healing, cutaneous inflammatory diseases, and epidermal barrier homeostasis is accumulating (Hachem *et al.*, 2006; Briot *et al.*, 2009). In the present study, we found a close relationship between PAR2, and Klk8 and Klk6 proteases. First, the induction of PAR2 expression after wounding was more marked in WT than Klk8$^{-/-}$ mice (Figure 5), suggesting that Klk8 may directly or indirectly induce PAR2. Second, PAR2 was expressed in the cytosol of keratinocytes in the stratum spinosum (Figure 5h and j). At the same time, Klk6 and Klk8 were localized in the intercellular spaces of the stratum spinosum and stratum granulosum (Figure 4h and j). Although Klk8 could not directly induce or activate PAR2, Klk8 may be involved in the activation of PAR2 via Klk6. PAR2 is thought to have a role in the terminal differentiation of keratinocytes based on the observation that cornification after acute skin barrier disruption was delayed in PAR2$^{-/-}$ mice (Demerjian *et al.*, 2008; Roelandt *et al.*, 2011). Possibly, Klk8 has a role in the switch between epidermal cell proliferation and differentiation through upregulation and activation of PAR2 at the late stages of wound healing, but the mechanism by which Klk8 modulates Klks-PAR2 signaling remains to be determined.

In conclusion, we showed that Klk8 is involved in wound healing associated with upregulation and activation of Klk6, and inducing keratinocyte differentiation possibly related to the upregulation of PAR2, although the mechanism of Klk6 and PAR2 upregulation remains to be determined. We propose that Klk8 regulates the balance between keratinocyte proliferation and differentiation in response to skin injury, and further understanding of its role in wound healing may reveal new therapeutic strategies in this area.

MATERIALS AND METHODS

Mice and wound healing model

All experiments were conducted with Klk8$^{-/-}$ mice with C57BL/6 genetic background (Hirata *et al.*, 2001) and WT C57BL/6 mice. All experimental protocols were carried out according to protocols

approved by the Institutional Animal Care and Use Committee of Asahikawa Medical University. Full-thickness, 8-mm punch biopsies were made on the shaved and depilated back of 8–12-week-old female WT and $Klk8^{-/-}$ mice. Wounds were left uncovered and harvested in its entirety along with a 5-mm margin at days 1, 3, 5, 7, and 14 post wounding. Specimens were frozen in liquid nitrogen and stored at −80 °C until use. In some mice, the maximum length of the wound was measured daily until complete epithelialization was achieved.

Reverse transcriptase–PCR

Total RNA was isolated according to the protocol of the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) for RT-PCR. A measure of 2 μg of total RNA was reverse transcribed with AMV reverse transcriptase (Promega, Madison, WI), and PCR was performed using the HotstarTaq DNA polymerase kit (Qiagen). Real-time quantitative PCR was performed following the protocol described in the Light cycler 480 SYBR I Master kit (Roche Diagnostic, Manheim, Germany), or Universal PCR master mix (Applied Biosystems, Foster City, CA). The primers and probe are listed in Supplementary Table S1 online. Results were represented using a standard curve for comparison or delta Ct method.

Histological observation

The wounded skin was fixed in 20% formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin.

For immunohistochemistry, 6-μm-thick frozen sections were incubated with primary antibody for 1 h at room temperature. Primary antibodies are listed in Supplementary Table S2 online. Primary antibody was detected using an FITC-conjugated or a peroxidase-conjugated secondary antibody. Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) was used for the detection of peroxidase. Additional information regarding the method of measurement for various factors is indicated in the Supplementary Material and Method online.

Western blotting

The incised mouse skin was incubated in 1 M NaCl at 4 °C for 72 hours. The epidermis was mechanically separated from the dermis and homogenized in a sample buffer containing 62.5 mM Tris-HCl, 2% glycerol, 1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Sigma, Saint Louis, MO), and centrifuged at 15,000*g* for 20 minutes at 4 °C. The supernatant was used as the epidermal extract. Proteins of the epidermal extract were separated by Novex Tris-Glycine gel (Invitrogen, Carlsbad, CA) SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated with primary antibodies. Primary antibodies are listed in Supplementary Table S2 online. The final detection was performed with 1:2000 dilutions of horseradish peroxidase–conjugated secondary antibodies and visualized using the ECL Advance Western Blotting Detection Kit (GE Healthcare UK, Buckinghamshire, UK), and detected by chemiluminescence using LAS-3000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan).

Statistical analysis

Student's *t*-test was used for statistical analysis. $P<0.05$ was considered statistically significant. Data are presented as mean ± SEM.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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